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Correlation of Tumor Necrosis Factor Levels in the Serum and Cerebrospinal Fluid With Clinical Outcome in Japanese Encephalitis Patients

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To investigate the prognostic role of tumour necrosis factor (TNF) in Japanese encephalitis virus (JEV) infection, we measured the immunoreactive forms of TNF concentrations in the serum and cerebrospinal fluid (CSF) of 47 laboratory-confirmed cases of JE. It was observed that TNF levels were elevated (>15 pgm/ml) in all the 47 serum samples (range 19.4–923.8 pg/ml), while in 46/47 CSF samples TNF was elevated (range 10.8–376 pg/ml). The mean (SD) TNF levels in the serum of fatal cases was 234.34 pg/ml (304.40) as compared to the mean of 85.31 pg/ml (SD 153.92) in nonfatal cases. Similar observations were also made with respect to the TNF levels in the CSF; the mean of fatal cases was 69.39 pg/ml (SD 39.00) in contrast to the mean of 62.41 pg/ml (SD 75.25) of nonfatal cases. The increase in TNF levels did not show any correlation to the duration of illness. It was further observed that the mortality rate increased with increasing concentrations of TNF in the serum and CSF. Correlation of laboratory parameters to final outcome revealed that TNF concentrations above 50 pg/ml in serum correlated significantly ($P = .05$) with a fatal outcome, whilst high levels of JEV-IgM antibodies (>500 units) in the CSF correlated with a nonfatal outcome ($P = .03$). These results suggest that TNF can be used as a possible prognosticator of a fatal outcome in JEV infection. ***J Med Virol 51:132–136, 1997.*** © 1997 Wiley-Liss, Inc.

KEY WORDS: Japanese encephalitis virus; cytokine; tumour necrosis factor; cerebrospinal fluid; outcome

INTRODUCTION

Japanese encephalitis (JE) is a mosquito-borne flavivirus infection of the human central nervous system. It is a major public health problem in several parts of Southeast Asia including India [Umenai et al., 1985]. The mortality in this acute encephalitic illness has varied from 20 to 40% during different epidemics in India with an average of 30% [Rodrigues, 1984]. The morbidity amongst survivors is also high, nearly half the survivors exhibiting neuropsychiatric sequelae [Gourie-Devi, 1984].

The role of tumour necrosis factor (TNF) in the host response to infection and injury is the subject of intense investigation. TNFα is a 17-kDa cytokine that is rapidly produced in response to infectious stimuli and appears to be pivotal for induction of other endogenous mediators as well as for initiation of several systemic responses to infection [Fong and Lowry, 1990]. In viral infections, TNF plays a key role in a variety of specific processes ranging from promoting viral replication and evasion of host defenses to regulating the humoral and cellular antiviral immune response [Campbell, 1991]. Consequently, this cytokine appears to be an important determinant of the clinical outcome during infection even with moderate release altering the immune and metabolic function in a manner appropriate to combating invading organisms. In contrast, exaggerated or prolonged secretion of TNF has been implicated in the pathogenesis of significant morbidity in diseases like cerebral malaria, septic shock, bacterial meningitis, leprosy, and other noninfectious conditions [Fong and Lowry, 1990; Grau, 1990]. However, there is very little information on the role of TNF in the evolution and pathology of acute viral encephalitides, especially Japanese encephalitis. In this study we measured TNF levels in the serum and CSF from laboratory-confirmed cases of Japanese encephalitis and attempted to correlate the levels of this cytokine with the clinical outcome.

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MATERIALS AND METHODS

Patients

This study was conducted on 47 patients admitted to the neurological services of the National Institute of Mental Health and Neurosciences Hospital between October and December 1991. The clinical examination and routine labortory investigations were suggestive of acute viral encephalitis. The diagnosis of Japanese encephalitis was established by the demonstration of either JEV-specific IgM antibodies in the CSF and/or JEV antigen in the CSF.

Samples

Serum (3 ml) and CSF (2 ml) was collected aseptically from all the patients on the day of admission to the hospital and transported immediately to the laboratory where they were aliquoted and stored at −70°C (within 30 minutes of collection).

JEV-Specific IgM Antibodies

An IgM antibody capture enzyme-linked immunosorbent assay (ELISA) described by Burke et al. [1985] was used with one modification. Biotinylated monoclonal antibody to the envelope protein of JEV was used instead of pooled flavivirus-reactive immunoglobulins fractionated from convalescent human subjects [Ravi et al., 1989a]. The results were expressed in IgM ELISA units. A sample was considered to be positive if it had >100 units in the CSF.

JEV Antigen Detection

A reverse passive haemagglutination described by Ravi et al. [1989a] was used for the detection of soluble JEV antigen in the CSF, while an immunofluorescent assay described by Mathur et al. [1990] was used for the detection of cell bound antigen in cytospin smears of the CSF cells.

TNF Estimation in Serum and CSF

Concentrations of TNFα in samples were determined by enzyme-amplified sensitivity immunoassay (EASIA) system with a sensitivity level of 1 pg/ml (Medgenix, Belgium). The lower and inner surface of the wells of the microtitre plate were coated with a mixture of monoclonal antibodies (an oligoclonal system), which recognise distinct epitopes of TNF. Recombinant TNF standards or test samples (100 μl) were added followed by 50 μl of incubation buffer. The plate was then incubated in a horizontal shaker for 2 hours. The plate was washed using 0.1% Tween 20 washing solution and aspiration. Monoclonal antibody (50 μl/well) recognising different epitopes of TNF and labelled with horseradish peroxidase was added to all the wells and incubated for 2 hours on a shaker at room temperature (RT). The plate was washed and reacted with freshly prepared tetramethylbenzene as chromogen in substrate buffer with H_2O_2 (200 μl/well) within 15 minutes of the washing step. The colour reaction was allowed to develop for 30 minutes on the shaker, avoiding exposure to direct sunlight. The reaction was stopped with sulphuric acid (50 μl/well), and the plate was read at 450 nm in the ELISA reader. The amount of substrate turnover was determined colorimetrically by measuring the absorbance which is directly proportional to the TNF concentrations. A standard curve was plotted with the readings obtained at 450 nm, and TNF concentrations in the samples were determined using the standard curve.

TABLE I. Distribution of TNF Levels in the Serum and CSF of Patients With Japanese Encephalitis (n = 47)

Range of TNF (pg/ml)	No. of sera	No. of CSF
<15	0	1
15–50	18	25
50–100	15	17
100–200	9	3
200–400	1	1
400–600	1	0
>600	3	0
Total	47	47

Statistical Methods

The correlational analysis between the clinical and laboratory parameters and the final clinical outcome was carried out using Fisher's test.

RESULTS

The diagnosis of Japanese encephalitis was confirmed in all 47 patients by demonstration of either virus-specific IgM antibodies in the CSF (28/47) or JEV antigen in the CSF (4/47) or the presence of both JEV-IgM antibodies and JEV antigen in the CSF (15/47). The mean age of the 47 patients in this study was 10.68 years (range 3–40, male:female ratio = 1.7:1). The mean duration of illness was 6.40 days (range 2–30), and in a majority (35/47) of the patients the CSF and serum samples were collected for analysis within the 1st week after onset of symptoms. The CSF cell counts were done in 30/47 patients, and the mean counts were 168.4 cells (range 5–800 cell/mm^3). Lymphocytic predominance was noted in the majority of these patients (22/30). Amongst 33/47 patients in whom the information regarding the final outcome was available, 12 patients succumbed to the infection while 21 recovered with (10/21) or without (11/21) neurological deficits.

The distribution of TNF levels in the serum and CSF samples obtained from the 47 JE patients is shown in Table I. The cytokine levels in the serum ranged from 19.4 pg/ml to 923.8 pg/ml (mean = 118.5 pgm/ml, SD = 171.5 pg/ml), while it ranged from 10.8 pg/ml to 376 pg/ml in the CSF (mean = 60.36 pg/ml, SD = 56.46 pg/ml). The TNF levels in the serum and CSF were equal in 7/47 patients (14.89%) in 34/47 patients (72.34%) it was higher in the serum as compared to the CSF, and in the remaining 6/47 patients (12.76%) CSF levels were higher than the serum levels. The TNF levels in the CSF did not bear any correlation to either the number or the type of inflammatory cells in the

TABLE II. Correlation of Clinical and Laboratory Parameters to Final Outcome in JE Cases (n = 33)†

Parameter evaluated	Fatal (n = 12)	Nonfatal (n = 12)	*P* value
Fever			
+	12	20	
−	0	1	(N/A)
Headache			
+	9	8	0.041
−	3	13	(SIG)
Vomitting			
+	8	9	0.188
−	4	12	(NS)
Convulsions			
+	11	14	0.106
−	1	7	(NS)
Altered sensorium[a]			
+	3	9	0.304
−	9	12	(NS)
CSF JEV-IgM			
>500 units	4	15	0.038
<500 units	8	6	(SIG)
Serum JEV-IgM[b]			
>500 units	2	12	0.051
<500 units	7	7	(NS)
CSF TNF Levels			
>50 pg/ml	7	8	0.447
<50 pg/ml	5	13	(NS)
Serum TNF Levels			
>50 pg/ml	9	7	0.050
<50 pg/ml	3	14	(SIG)

†+, presence of the variable; −, absence of the variable; N/A, statistics not applicable; SIG, significant; NS, not significant.
[a]This was scored as presence (+) or absence (−) of response to either verbal commands and/or painful stimuli.
[b]In five patients serum JEV-IgM could not be measured and amongst them three were in the fatal group and two were in the nonfatal group.

CSF during the course of the illness. Amongst the 47 patients, TNF estimations in serum and CSF were done in 35 during the 1st week after onset of symptoms, in five patients during the 2nd week after onset of symptoms, in two during the 3rd week, and in one during the 4th week. In 3/47 patients the precise date of onset of symptoms could not be ascertained. The levels of TNF in the serum and CSF did not show any correlation to the duration of illness. For instance, high levels of TNF (>100 pg/ml) were noted in 6/15 sera tested early in the course of the illness (<4 days after onset of symptoms), whilst low levels were seen late in the illness. CSF TNF levels also did not reveal any correlation to the duration of illness excepting that the highest TNF value (376 pg/ml) was obtained in one patient who survived the illness and was tested on the 30th day after onset of symptoms.

Table II presents the correlation of clinical and laboratory parameters to the final outcome in 33 patients. None of the clinical parameters evaluated other than the presence of headache (at the onset of illness) were able to serve as a prognosticator. Amongst the laboratory parameters high levels of TNF in the serum (>50 pg/ml) correlated with a fatal outcome, whilst high levels of JEV-IgM antibodies (>500 units) in the CSF correlated with a nonfatal outcome. It was also observed that the mortality increased with increasing amounts of TNF in serum as well as CSF (Fig. 1). At serum TNF concentrations of less than 50 pg/ml the mortality was 17%, while it increased to 55% when serum concentrations were between 50 and 100 pg/ml and 57% at serum concentrations more than 100 pg/ml. A similar pattern was also observed with respect to the CSF concentrations and the mortality (Fig. 1). The correlation of laboratory parameters to outcome in the 33 patients was also attempted by dividing the patients into the three viz. recovered, sequelae, and fatal groups (data not presented). However, no meaningful conclusions could be drawn from such an analysis probably because of the few numbers in each group.

DISCUSSION

Tumour necrosis factor alpha (TNF) is widely recognised as a central endogenous mediator of inflammation in several human diseases [Grau, 1990]. There is sufficient evidence now to indicate that TNF has a central role in the pathophysiology of septic shock, cerebral malaria, and bacterial meningitis [Fong and Lowry, 1990; Grau, 1990; Feldman, 1991]. For instance, it has been observed by Grau et al. [1989] that elevated serum

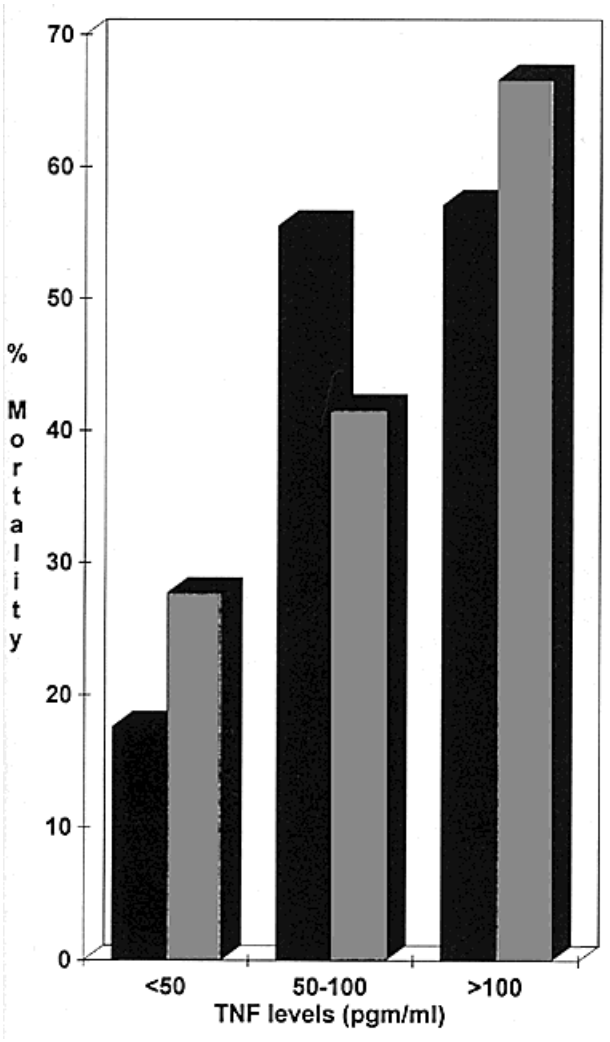

Fig. 1. Serum (dark bars) and CSF (grey bars) TNF levels in Japanese encephalitis patients (n = 33) at the time of admission to the hospital in relation to the clinical outcome. Percentage mortality was higher in patients whose TNF levels were greater than 50 pg/ml.

levels of TNF correlate with death in Malawian children with *Plasmodium falciparum* malaria. They also observed that the levels of TNF at the time of hospital admission were significantly higher in children with neurological manifestations as compared to those without neurological impairment. Although the traditional role of a mediator in the local and systemic antiviral response has been ascribed, to TNF it is known to promote the replication and spread of certain viruses, especially the HIV [Campbell, 1991]. However, with respect to JEV, it has been observed in an in vitro study that TNF does not influence the viral titres in the infected cultures of human monocytes [Hasegawa et al., 1990]. Hence, it is difficult to predict the direct effect of TNF on JEV replication. On the other hand, TNF is known to contribute to a variety of inflammatory processes in the CNS ranging from demyelination, cytotoxic damage to endothelium, and necrosis of oligodendrocytes to interfering with the propagation of nerve impulse [Hartung, 1993]. These observations prompted us to investigate the role of TNF in patients with JE and to evaluate its role in influencing the morbidity and mortality of the illness.

In the present study we have estimated the levels of immunoreacive TNF in the serum and CSF of 47 confirmed cases of JE. The TNF levels were elevated (>15 pg/ml) in all the serum samples and 46/47 CSF samples. In 6/47 patients TNF levels were greater in the CSF than in serum, probably indicating "local production" within the CNS. This is not a surprising finding since similar observations have been made in other CNS infections wherein TNF levels were greater in the CSF compartment than the serum [Moller et al., 1991]. The probable explanation for this being TNF production is not restricted to mononuclear cells alone in the CSF, and astrocytes are known to secrete this cytokine [Moller et al., 1991]. This probably also accounts for the lack of a correlation between the TNF levels in the CSF and the cell counts observed in this study.

Until recently the only risk factor contributing to a fatal outcome in JE patients at the time of admission into the hospital has been the presence of virus in the CSF [Burke et al., 1985]. However, recent studies on the immune response to JEV in our laboratory have indicated that the presence of autoantibodies to neural antigens and virus-specific immune complexes in the CSF are also poor prognosticators of outcome [Desai et al., 1994, 1995]. The presence of JEV-specific IgM antibodies in the CSF on the other hand has been correlated to a nonfatal outcome [Burke et al., 1985; Ravi et al., 1989b]. Similarly, in the present study also the presence of high levels of JEV-IgM in the CSF has emerged as a prognosticator of nonfatal outcome. In addition the measurement of TNF levels in the serum and CSF of JE patients has emerged as a useful prognosticator in predicting the fatal outcome of JE cases. Our results here suggest that the elevated levels of TNF in the serum parallel the increase in the mortality of JE patients (Fig. 1). Similar patterns were also observed with respect to the CSF concentrations and the mortality, although the results were not statistically significant ($P = .447$). It is noteworthy that the elevated TNF levels in serum and CSF did not show any correlation to the duration of illness. This suggests that the increase in TNF levels observed was probably a direct reflection on the underlying inflammatory process rather than on the duration of illness. It must be noted here that we have estimated only the immunoreactive forms of TNF. Therefore, it is likely that binding proteins or inhibitors would modulate the bioactivity of this cytokine.

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